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Effect of temperature on protein quality in bacterial inclusion bodies

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Abstract **Increasing evidence indicates that protein aggregation in bacteria does not necessarily imply loss of biological activity. Here, we have investigated the effect of growth-temperature on both the activity and stability of the inclusion bodies formed by a point-mutant of Aβ42 Alzheimer peptide, using green fluorescent protein as a reporter. The activity in the aggregates inversely correlates with the temperature. In contrast, inclusion bodies become more stable in front of chemical denaturation and proteolysis when temperature increases. Overall, the data herein open new perspectives in protein production, while suggesting a kinetic competition between protein folding and aggregation during recombinant protein expression.**

Keywords: Inclusion bodies; Protein aggregation; Protein folding; Recombinant protein expression; *Escherichia coli*

1. Introduction

In many cases, the production of recombinant polypeptides in prokaryotic hosts results in incomplete folding processes that usually end with their accumulation as insoluble aggregates, known as inclusion bodies (IBs), in the cytoplasm and/or in the periplasmic space of the cells [1–3]. The aggregation of insoluble polypeptide chains as IBs is of major concern in biotechnology, since it prevents the commercialisation of many relevant polypeptides [3]. The complete aggregation process is still poorly understood. The current view about IBs has recently evolved from considering proteins in IBs as totally inactive to accept that aggregation of recombinant proteins as bacterial IBs does not necessarily inactivate them [4–7]. This allows using the activity of the protein embedded in IBs as a reporter to monitor the influence of both intrinsic and extrinsic factors on the aggregation process. This could become an important subject in biotechnology since it can be of help in tuning optimal sequential and culture conditions for protein production.

We have shown that, the overexpression of a fusion of the aggregation-prone, Alzheimer-related, peptide Aβ42 to green fluorescent protein (GFP) results in fluorescent IBs. Using this system we have investigated the effect of the polypeptide sequence on protein quality in bacterial aggregates [8]. The approach also permits to easily monitor the effects of extrinsic factors, such as growth temperature, on bacterial protein aggregation.

A widespread strategy to reduce the in vivo aggregation of recombinant polypeptides consists of cultivation at reduced temperatures [9]. This approach has proven effective in increasing the solubility of a number of difficult proteins at expenses of the final yield [10]. However, only recently it has been addressed the effect of the temperature on the characteristics of the aggregated fraction [5,11–13]. In the present investigation, we have quantitatively investigated the biological activity and the stability of the IBs formed by a variant of the Aβ42-GFP fusion protein at different cultivation temperatures to provide insights into the rules and polypeptide interactions underlying protein deposition during recombinant protein expression.

2. Material and methods

2.1. Protein expression and IBs purification

Escherichia coli BL21(DE3) was used for all the experiments. Plasmid encoding Aβ42(F19D)-GFP has been previously described [8]. Cells expressing the Aβ42-GFP fusion were grown for 4 h at 37 °C in LB medium containing 35 μg/ml kanamycin and pre-incubated at the selected expression temperature for 30 min. Then, protein expression was induced with 1 mM IPTG. Cultures were grown at the selected temperatures for 20 additional hours, to ensure fluorescence equilibrium, and harvested by centrifugation. Expression of Aβ-GFP fusion protein was monitored by SDS–PAGE using a 12% (w/v) gel. Inclusion bodies (IBs) were purified from cell extracts by detergent-based procedures as described [14]. For the determination of inclusion body protein, these structures were resuspended in denaturing buffer [15]. After boiling for 10 min, appropriate sample volumes were loaded onto denaturing gels. Gels were scanned at high resolution and bands quantified by using the Quantity One software from Bio Rad, by employing appropriate protein dilutions of known concentration as controls. Determinations were always done within the linear range and they were used to calculate the specific activity values.

2.2. Fluorescence measurements

Emission spectra of GFP in IBs were measured on a Perkin–Elmer 650-40 spectrofluorimeter (Boston, MA, USA). The GFP fluorescence of a 1 ml of IBs suspension in 10 mM Tris–HCl buffer (pH 7.5) was recorded from 500 to 600 nm, using an excitation wavelength of 470 nm. Emission and excitation slits width were fixed at 10 and 5 mm, respectively. Dilutions were employed when necessary and data were corrected for buffer signals and protein concentration. At least three different scans were averaged for each IBs sample. For microscopy analysis, IBs formed at different temperatures were isolated from the insoluble cell fraction by repeated detergent washing as described [14] and deposited on top of glass slides. Images of purified IBs were obtained at 40-fold magnification under UV light or using phase contrast in a Leica DMBR microscope. The average size of purified IBs was measured under phase contrast by analysing 40 individual aggregates corresponding to two different fields for each temperature, using the Leica QWin Standard V2.3 software.

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2.3. Proteolytic digestion of IBs

Purified Aβ42(F19D)-GFP IBs, obtained at different temperatures, were passed 10 times through a 0,1 mm needle to homogenize the aggregate solutions. The IBs were diluted at 1 $OD_{350\,nm}$ in 792 μl of 50 mM Tris–HCl, 150 mM NaCl buffer (pH 8.0). 8 μl of concentrated proteinase K was added the IBs solution to obtain a 0.2 mg/ml final concentration and initiate the proteolytic reaction. The digestion was monitored for 150 min by measuring the changes in $OD_{350\,nm}$ in a Cary-100 Varian spectrophotometer.

2.4. Stability of IBs in front of chemical solubilization

50 μl of a 1 $OD_{350\,nm}$ solution of purified and homogenized Aβ42(F19D)-GFP IBs, obtained at different temperatures, was added to 950 μl of 10 mM Tris–HCl buffer (pH 7.5) containing selected concentrations of guanidinium hydrochloride (ranging from 0 to 6 M) for equilibrium denaturation experiments. The reactions were allowed to reach equilibrium by incubating them for 20 h at room temperature. The effect of the denaturant was measured by monitoring the changes in $OD_{350\,nm}$ in a Cary-100 Varian spectrophotometer. The fitting of the experimental data was performed using the non-linear, least-squares algorithm provided with the software KaleidaGraph (Abelbeck Software) assuming a two-state solubilization mechanism.

For kinetic experiments, 50 μl of a 1 $OD_{350\,nm}$ solution of purified and homogenized Aβ42(F19D)-GFP IBs, obtained at different temperatures, was added to 950 of 10 mM Tris–HCl buffer (pH 7.5) containing 2,3 M guanidinium hydrochloride. The reaction was monitored for 180 min by measuring the changes in $OD_{350\,nm}$ in a Cary-100 Varian spectrophotometer. Double-exponential decay curves were fitted to the data using Sigmaplot non-linear regression software (Jandel Scientific, San Rafael, CA, USA), and apparent rate constants were derived from these regressions.

3. Results and discussion

3.1. Effect of the growth temperature on the activity of IBs

In a previous study we have used the Alzheimer related Aβ42 gene fused upstream of the GFP sequence and under the control of the T7 promoter as a model to investigate aggregation in bacteria. At 37 °C, *E. coli* cells transformed with this construction express, upon IPTG induction, a high amount of the Aβ42-GFP fusion protein that accumulates in active fluorescent cytoplasmatic inclusion bodies [8]. We have shown that mutation of Phe in position 19 of Aβ42 to Asp abolish the amyloidogenicity of this Alzheimer-related peptide [16]. This change also promotes a fourfold increase in the specific fluorescence emitted by IBs relative to that emitted by the wild type Aβ42 when fused to GFP and expressed at 37 °C in *E. coli* [8]. This mutant provides a wider dynamic range to explore the effects of extrinsic factors on the fluorescence of IBs, especially if conditions expected to decrease the activity of these aggregates are going to be tested. In the present study we explored whether the temperature of cultivation influences the activity of the protein embedded in the aggregates or if on the contrary the fluorescence of the IBs is independent of the temperature at which they are formed. To this aim we expressed the Aβ42(F19D)-GFP fusion at temperatures ranging from 18 to 42 °C. A fraction of the expressed protein fusion accumulated as insoluble IBs at all the temperatures assayed. We purified the different inclusion bodies and compared their specific activity by measuring GFP fluorescence emission. As shown in Fig. 1 the activity of the protein in IBs is strongly influenced by the temperature of cultivation. Increasing the growth temperature above 37 °C results in reduction in specific activity, while lowering it significantly increases the fluorescence emission. The specific fluorescence of the IBs formed at 18 °C was 16-fold higher than those exhibited by the IBs

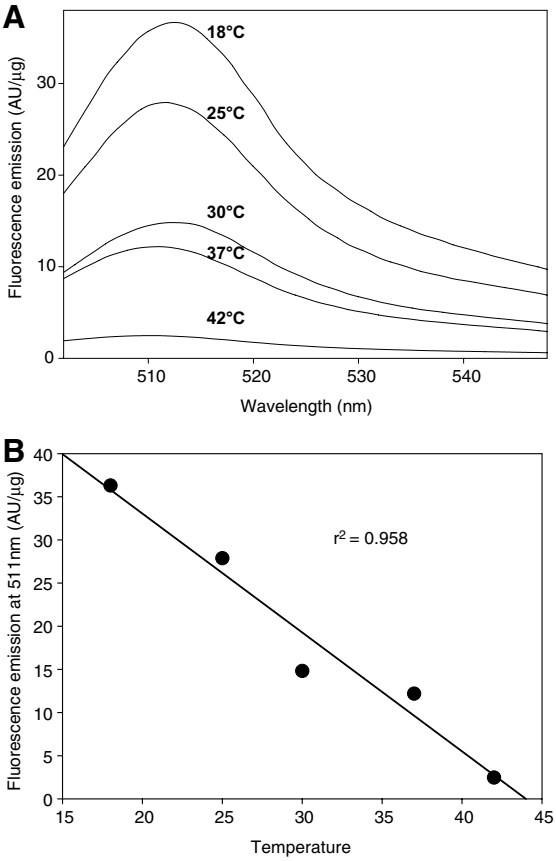

Fig. 1. Dependence of the specific fluorescence emission of Aβ42(F19D)-GFP IBs on the growth temperature. (A) Fluorescence spectra of GFP in IBs at selected temperatures. (B) Correlation between IBs activity and temperature of cultivation.

purified from cells cultured at 42 °C. The influence of temperature on IBs fluorescence emission, once purified from the insoluble cell fraction, can be also visually analyzed by using fluorescence microscopy (Fig. 2). In agreement with the spectral data, IBs formed at low temperatures appear clearly as a more fluorescent aggregates than those formed at high temperature. Using phase contrast it could be observed that the isolated aggregates formed at 42 °C, 37 °C, 30 °C, and 25 °C all display similar sizes, ranging from 1.1 to 1.2 μm. The IBs formed at 18 °C appear as smaller, much less refractile aggregates with an average size of about 0.9 μm. Interestingly, similar morphological and fluorescent properties have been reported recently for the aggregates formed by a VP1-GFP fusion at 16 °C [12]. In order to rationalize how temperature promotes changes in IBs activity, we studied the correlation between IBs specific fluorescence and the cultivation temperature. A strong inverse correlation was observed indicating a linear dependence of IBs activity on the growth temperature in the studied range (Fig. 1B) and thus an increase in the proportion of native-like conformations in IBs formed at low temperatures.

3.2. Effect of the growth temperature on the stability of IBs

It is thought that during recombinant protein production aggregation is in general favoured at higher temperatures due to the strong temperature dependence of hydrophobic

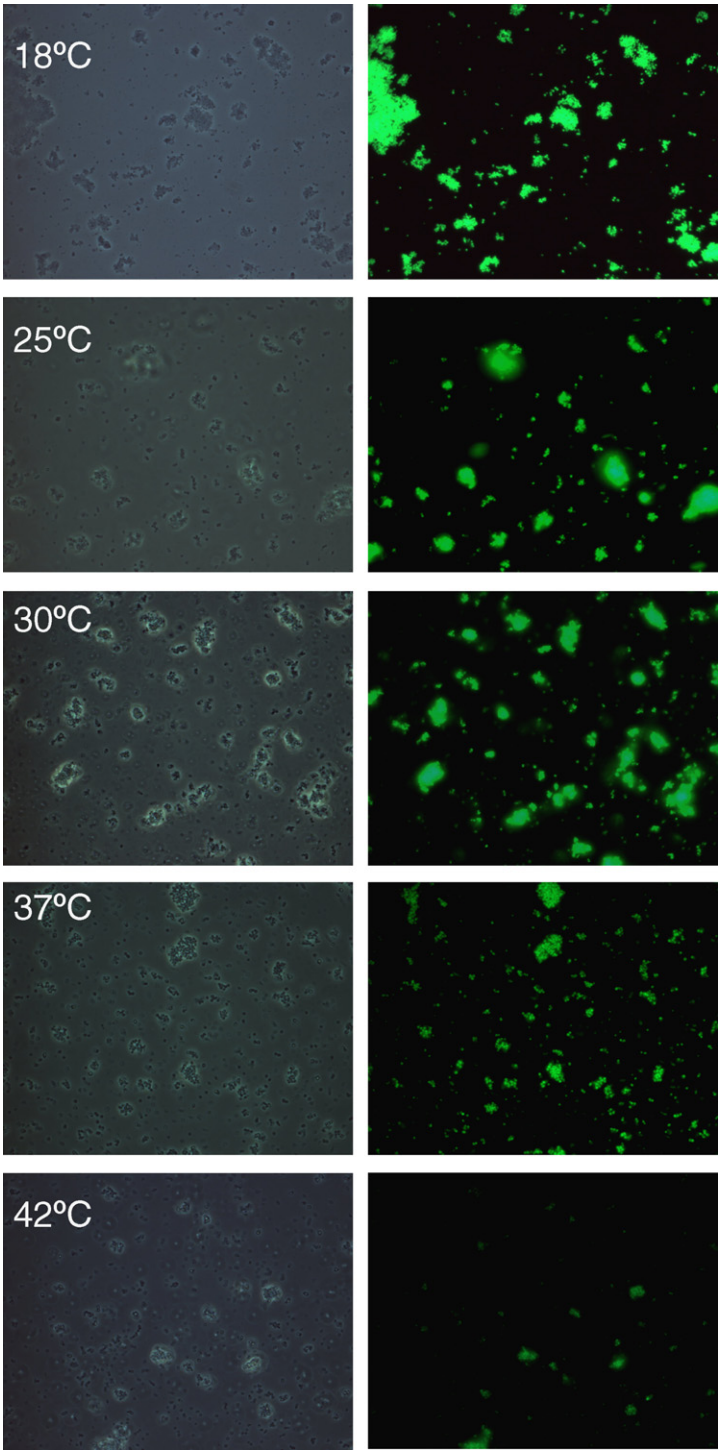

Fig. 2. Visualization of GFP fluorescence in isolated IBs formed at different temperatures. The left series correspond to phase contrast microscopy of purified IBs, the right series to fluorescence microscopy under UV light, both with 40-fold magnification.

interactions involved in the aggregation reaction [17,18]. Nevertheless, and to the best of our knowledge, it has not been investigated yet whether this results in a dependence of IBs conformational stability on the growth temperature. To explore this possibility, we compared first the resistance to proteinase K digestion of the IBs formed under standard conditions (37 °C) with that of IBs formed at higher (42 °C), and lower (25 °C) temperatures. Proteinase K is an endolytic serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. It has find application in the mapping of polypeptide regions in the core of amyloid fibrils due to its strong preference for hydrolyzing unstructured protein regions [19]. We monitored the kinetics of IBs sensitivity to proteolysis by measuring the decrease in turbidity at 350 nm upon addition of proteinase K. As illustrated in Fig. 3, IBs formed at high temperature

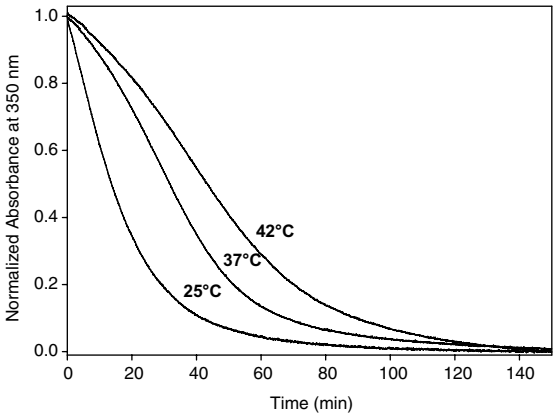

Fig. 3. Kinetics of IBs' proteolytic digestion monitored by a time-dependent decrease of turbidity at 350 nm. The growth temperatures at which inclusion bodies were obtained are indicated on top of the curves.

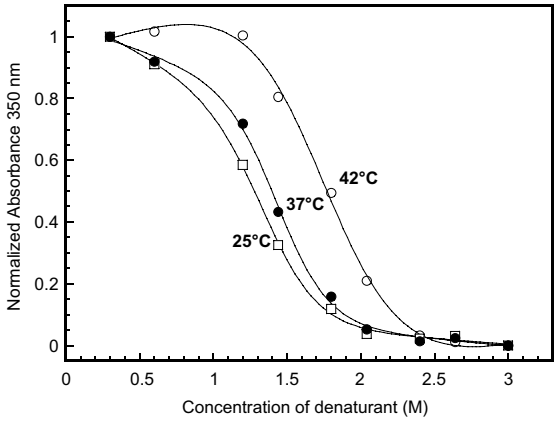

Fig. 4. Stability of IBs in front of Gnd HCl denaturation under equilibrium conditions. The growth temperatures at which inclusion bodies were obtained are indicated on side of the curves.

are clearly more stable in front of proteolysis than those formed at low temperature. The IBs formed at 42 °C and 37 °C exhibited a sigmoid curve that suggests an initial higher resistance to digestion, and thus a more densely packed structure in the initial aggregated species that is lost after the initial proteolytic attack. This effect is not appreciable in the IBs formed at 25 °C, indicating a high sensibility to protease action already at the beginning of the experiment, probably due to the presence of a higher amount of accessible polypeptide chains.

We investigated if the differential stability of these IBs in front of proteases correlates with their resistance in front of chemical denaturation. To this aim, we dissolved the different IBs in buffer containing selected concentrations of guanidinium hydrochloride (Gnd HCl). This chaotropic agent has been used recently to study the resistance to solubilization of the IBs and thermal aggregates formed by different proteins [20]. The reaction was typically performed at room temperature for 20 h to allow equilibrium; then, the effect of the denaturant was measured by monitoring the changes in absorbance at 350 nm. We assumed a two-state mechanism in which the protein is either in an aggregated state that contributes to turbidity or in a soluble state which does not contributes to the absorbance at 350 nm (independently of the fact that the protein could be properly folded or not in the aggregated or soluble states). Although this assumption is clearly a simplification of the effect of the chaotropic agent on IBs, the curves could be properly fitted to a two states process ($R = 0.999$ in all cases) (Fig. 4). From the data it can be clearly inferred that IBs formed at different temperatures differ also in their conformational stability against chemical denaturation, being again the IBs formed at 42 °C (transition midpoint = 1.72 M Gnd HCl) more tolerant to the presence of Gnd HCl than those formed at 37 °C (transition midpoint = 1,45 M Gnd HCl) or 25 °C (transition midpoint = 1,36 M Gnd HCl). To further confirm this point we sought to analyze the kinetics of solubilization of the three different IBs by a fixed concentration of denaturant. This way, we incubated the IBs formed at 42 °C, 37 °C and 25 °C in 2,3 M Gnd HCl and monitored the dependence of the turbidity signal at 350 nm on the time (Fig. 5). The data could be fitted to a double-exponential decay equation with very good accuracy ($R > 0.99$) and the differences in the apparent rate constants of the fast phase calculated. Significant differences in the velocity of solubilization could be observed between samples, with 0.376 ± 0.005, 0.290 ± 0.002 and $0.219 \pm 0.002\ \text{min}^{-1}$ fast rate constants for IBs formed at 25 °C, 37 °C and 42 °C, respectively. Thus, in excellent agreement with the equilibrium data, the IBs formed at low temperatures are solubilized faster than those formed at higher temperatures, indicating that the cultivation temperature determines the stability, and thus the conformational properties of the polypeptide chains embedded in bacterial aggregates.

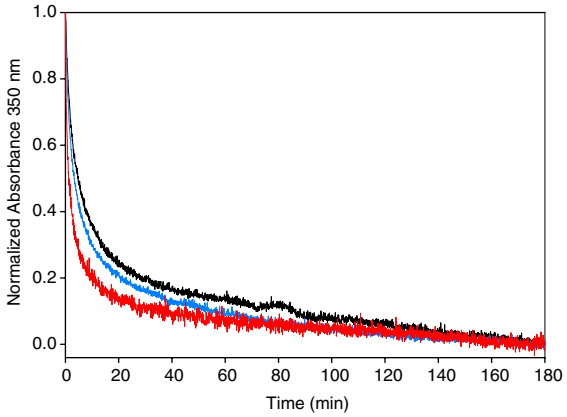

Fig. 5. Kinetics of solubilization by 2,3 M Gnd HCl of IBs formed at 42 °C (black), 37 °C (blue) and 25 °C (red), monitored by a time-dependent decrease of turbidity at 350 nm.

3.3. Relationship between IBs conformational stability and activity

To decipher if the solubilization of IBs by chemical denaturation affects the activity of the protein embedded in these bacterial aggregates, we investigated the effect of Gnd HCl on IBs activity and compared it with the impact on IBs conformational stability. We proceeded as described for the equilibrium denaturation experiment, but this time we monitored the dependence of GFP fluorescence emission on denaturant concentration. In Fig. 6 the equilibrium curves obtained by monitoring absorbance at 350 nm and protein fluorescence in IBs

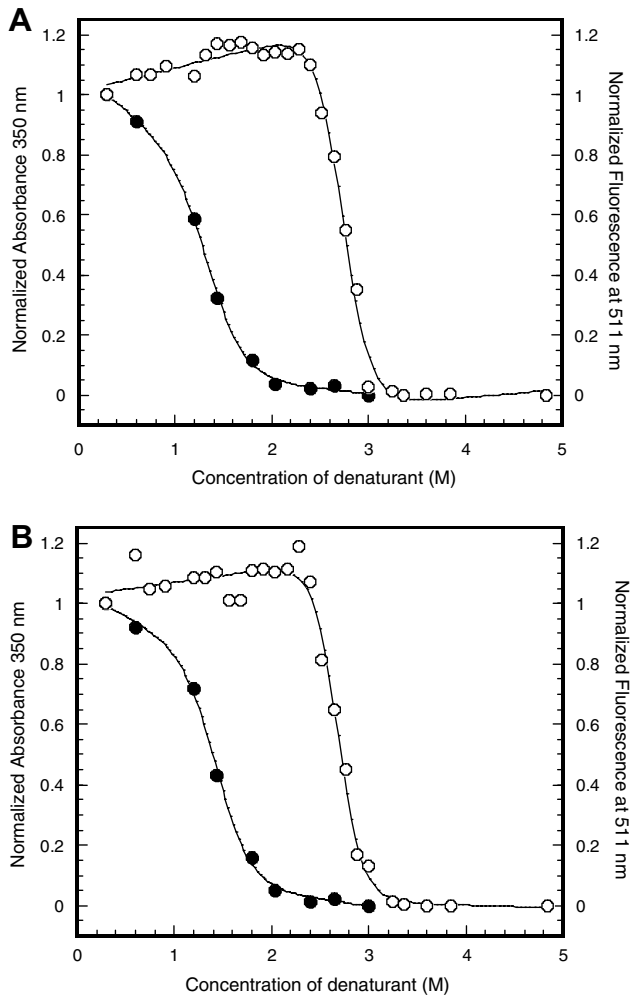

Fig. 6. Equilibrium dependence of the turbidity signal (solid symbols) and GFP fluorescence (empty symbols) of IBs formed at 25 °C (A) and 37 °C (B) on the Gnd HCl concentration.

growth at 25 and 37 °C are compared (the low activity of IBs formed at 42 °C prevented to record accurate data at high denaturant concentrations). Surprisingly, it is observed that the turbidity signal disappears at chaotropic agent concentrations in which GFP is still fully active in both types of IBs, indicating that the turbidity curve provides us mainly information about the loss of the intermolecular interactions that stabilize IBs rather than on intramolecular contacts, which would account for the native conformation and activity of GFP. This results has important implications for recombinant protein production since suggests that proteins can be liberated from IBs in a fully functional state, by using conditions which specifically disturb the network of intermolecular contacts that provides stability to IBs without denaturating the native protein embedded in the aggregates. These data are in agreement with the suggestion that the presence of native-like structures within IBs could improve the efficiency of refolding strategies that use mild solubilization conditions [21], as well as with the observation that the in vitro refolding of IBs formed at low temperature renders higher yields of active polypeptides than when employing IBs constructed at higher temperatures [22].

From the present data, it follows that high temperatures promote stable aggregates because they favour intermolecular interactions at expenses of native intramolecular contacts and thus protein activity. This way the lower activity of IBs produced at high temperature indicates a higher proportion of non-native protein conformations respect to IBs formed at low temperature. In principle, this non-properly folded polypeptide chains or segments are ready to establish intermolecular interactions among them in the aggregates promoting their stability. These contacts are more likely involved in the formation and stabilization of the intermolecular β-sheet structure recently described to be common to IBs formed by unrelated proteins [23–25]. On the contrary, production of protein at low temperatures results in highly active IBs which indicates that in this molecules, aggregation-promoting regions are likely to be blocked in the native state of globular GFP because their side chains are hidden in the inner hydrophobic core or already involved in the network of contacts that stabilizes the native state of a protein [26]. The lower number of unfolded, aggregation-prone chains available to establish the intermolecular interactions that glue the structure of IBs would explain the lower conformational stability of these low temperature aggregates. It is also deduced that, at least in this particular case, the intramolecular native contacts that maintain the folded protein structure are stronger than non-native interactions between polypeptide chains, since they resist clearly higher denaturant concentrations. Interestingly, Villaverde and co-workers have recently reported, using GFP and a VP1-GFP fusion, that, in excellent agreement with the present data, low temperature cultivation results in more active IBs [12]. Although the stability of the different IBs was not addressed in this work, it was demonstrated that the intermolecular extended β-sheet conformation of IBs loosed compactness at lower temperatures as demonstrated by ATR-FTIR. Similar structural results have been found for recombinant growth hormone, human interferon-alpha-2b and a lipase when expressed in *E. coli* as IBs [5,27]. In these particular cases, the relative intensity between the native and the aggregated IR contributions was shown to be modulated by protein expression levels. Overall, this conjunct of recently collected data provides evidence that during protein recombinant production the processes of folding to attain a native conformation stabilized by intrachain contacts and aggregation to attain a non-native structure stabilized by interchain interactions are competing. As shown here, for a given polypeptide, the equilibrium can be shifted in either direction by specific extrinsic factors.

An increasing body of evidence [1,28,29] suggests that the possibility to find out strategies that favour in vivo folding versus aggregation would open intriguing opportunities both in the protein production and protein aggregation research. This way, the production in bacteria of highly active and poorly stable IBs by modulating the culture conditions, together with the development of simple and economic downstream strategies, such us mild IBs disaggregation (without the requirement for aggressive unfolding/refolding steps) appears as a promising avenue for the production of difficult proteins, such us mammalian ones, in a soluble, properly folded and active conformation useful for biotechnological applications.

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